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A study of polymethoxy-1-alkenes in *Raphidiopsis (Cylindrospermopsis) raciborskii* and *Aphanizomenon gracile* isolated in Poland

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Abstract

Previous studies indicated that teratogenic polymethoxy-1-alkenes (PMAs) are produced by phylogenetically diverse cyanobacteria taxa, **however corresponding studies on the occurrence of PMAs in European cyanobacteria is lacking**. Herein, the **presence of PMAs in strains of *Raphidiopsis raciborskii* and *Aphanizomenon gracile* isolated from surface waters in Poland was studied** using **nuclear magnetic resonance** and mass spectrometry. No PMAs were detected **in any of the strains investigated**, indicating that production of these compounds **may be** geographically diversified. Further studies are necessary to elucidate mechanisms of cyanobacterial PMAs synthesis.

Keywords: Polymethoxy-1-alkenes; Cyanobacteria; *Cylindrospermopsis raciborskii*; *Aphanizomenon gracile*

The toxicity of cyanobacteria attracts ongoing attention, and over the past few decades, the structures of numerous toxic metabolites have been identified, their biosynthesis pathways elucidated and their potential ecological roles proposed. The most extensively studied cyanobacterial compounds include the hepatotoxic cyclic peptides, microcystins and nodularins, cytotoxic alkaloid cylindrospermopsin, the neurotoxic alkaloids saxitoxins and anatoxin-a, and more recently a neurotoxic amino acid β -methylamino-l-alanine (Poniedzialek et al. 2012; Rzymiski et al. 2014; Testai et al. 2016; Buratti et al. 2017; Chernoff et al. 2017). In general, production of cyanotoxins has been found to be a species and strain-specific feature, and seems to be geographically diversified. Despite the extensive research, a significant number of cyanobacteria have shown *in vivo* and *in vitro* toxicity (including human cells), however the metabolites responsible for this activity were not identified. This is of particular concern for European strains belonging to the Nostocales order such as *Raphidiopsis* (*Cylindrospermopsis*) *raciborskii* and some members of the traditional “*Aphanizomenon*” genus (Bernard et al. 2003; Fastner et al. 2003; Antal et al. 2011; Acs et al. 2013; Poniedzialek et al. 2015; Vehovszky et al. 2015; Rzymiski et al. 2017; Falfushynska et al. 2019), and highlights the need for further explorations in this area.

Polymethoxy-1-alkenes (PMAs) represent a group of lipophilic compounds and varying in chain length (Fig. 1). Initially, PMAs were identified in *Tolypothrix conglutinate* (Mynderse and Moore 1979), *Scytonema ocellatum* (Mori et al. 1991a, b) and later, in an Israeli strain of *Aphanizomenon ovalisporum* (Banker et al. 2000), an Australian strain of *R. raciborskii* and other cyanobacterial species (Jaja-Chimedza et al. 2015). There is, however no information on the production of PMAs in any cyanobacteria occurring in European surface waters. Considering that PMAs may exert significant teratogenic action by triggering developmental dysfunctions (as presented using the zebrafish (*Danio rerio*) embryo model; Jaja-Chimedza et

al. 2012, 2015)), it is imperative to evaluate whether cyanobacteria strains previously shown to be toxic are producing these metabolites.

In this study the presence of PMAs in strains of *Aphanizomenon gracile* and *R. raciborskii* isolated from Western Poland was tested using nuclear magnetic resonance (NMR) and mass spectrometry (MS). These strains do not produce cylindrospermopsin (CYN) (although a CYN producing strain of *A. gracile* has been reported in Poland; see Kokociński et al. 2013), microcystins, saxitoxins, anatoxin-a, β -methylamino-L-alanine or diaminobutyric acid (with exception of *A. gracile* LWI-Ag in which it was found at levels below the level of quantification). However, selected extracts of these strains were found to exhibit relevant toxicities in human and fish cells *in vitro* (Poniedziałek et al. 2015; Rzymiski et al. 2017a; Falfushynska et al. 2019). This, in turn, highlighted the necessity to investigate whether any of these strains is capable of producing PMAs (Rzymiski et al. 2017b).

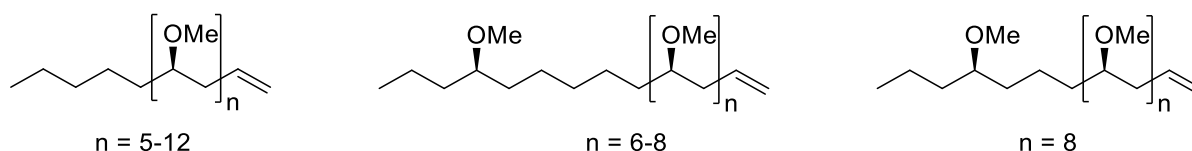


Figure 1. Chemical structure of known PMA variants.

Three strains of *R. raciborskii* and four strains of *A. gracile* (Fig. S1) were isolated from water samples from freshwater lakes in Western Poland (Table 1). Single trichomes were isolated under light and inverted microscopes using elongated glass Pasteur pipette and transferred to sterile WC medium prepared according to Guillard and Lorenzen (1972). This procedure was repeated until cyanobacterial monocultures were obtained. All cultures were maintained to stationary phase reaching 10^6 trichomes L^{-1} and then processed for analysis. All strains of *R. raciborskii* had characteristic morphological features for this species *viz* solitary trichomes, with straight cylindrical cells which are constricted at the cross-walls, together with

terminal heterocytes at one or both ends of the trichomes with a characteristic elongated drop-lake shape. Apical cells were clearly narrowed if heterocytes were not present. *A. gracile* similarly had characteristic morphological features viz solitary trychomes, with straight cylindrical cells constricted at the cross-walls together with often elongated and narrowed terminal cells with intercalary and solitary barrel-like shape heterocytes. Cylindrical, elongated akinetes with a characteristic cup-shaped sheath formation were rarely observed under culture conditions.

The PMAs were analyzed using two methods and initially, NMR analysis was employed. The analysis was targeted towards the identification of known PMAs and also the presence of potential homologous compound which high contain extra $[-CH=CH-]_n$, $[-CH_2CH(OMe)-]_n$ or $[-CH_2CH_2-]_n$ fragments. The biomass of each strain was collected by centrifugation and ultrasonicated on ice for complete lysis as previously described (Rzymiski et al. 2017a). The obtained samples were evaporated to dryness using a rotary evaporator (Buchi R124 Rotavapor) and the residue dried under high vacuum. 1H NMR spectra of the $CDCl_3$ soluble and D_2O soluble material from the residue were recorded using a Bruker Ultrashield Plus 400 MHz instrument. Inspection of the regions 5.00-6.00 ppm (vinylic protons) and 3.00-3.70 ppm ($MeOCH$, methyl and methine protons) did not clearly indicate the presence of PMAs on comparison with literature NMR data since no analytical standards were available and no database for these compounds exist (Mori et al. 1991a, Mori et al. 1991b, Mynderse and Moore 1979). In order to determine if any of the known PMAs were present in the residues obtained, but below the detection limits of NMR analysis, the samples were further investigated by mass spectrometry.

Table 1. Polish strains of *R. raciborskii* and *A. gracile* screened for PMAs production in this study.

Species/Strain	Place of isolation	Coordinates
<i>Raphidiopsis raciborskii</i>		
LBO-Cr	Lake Boczowskie	52°19'10"N, 14°56'47"E
LBY-Cr	Lake Bytyńskie	52°29'55"N, 16°30'30"E
LBU-Cr	Lake Buszewskie	52°32'34"N, 16°22'38"E
<i>Aphanizomenon gracile</i>		
LWI-Ag	Lake Witobelskie	52°15'55"N, 16°43'32"E
LPN-Ag	Lake Pniewskie	52°30'41"N, 16°14'27"E
LBN-Ag	Lake Bnińskie	52°12'02"N, 17°06'59"E
LJE-Ag	Lake Jelonek	52°32'04"N, 17°35'17"E

96

97 To this end, a separate set of sample of cyanobacterial biomass was collected by
98 centrifugation, freeze-dried, then suspended in CHCl₃, stirred vigorously for 16 h then filtered
99 through a sintered filter apparatus. This procedure was repeated a total of three times and the
100 combined filtrates were dried over anhydrous magnesium sulphate and the solvent removed
101 under reduced pressure. The residue obtained was then dried under high vacuum until constant
102 weight. The material obtained was fractioned using silica gel chromatography eluting with a
103 stepwise gradient of ethyl acetate in petrol (10%, 20%, 40% and 100%). Each fraction was
104 collected and evaporated to dryness. MS analysis was performed either on a Xevo G2-S QTof
105 or a LTQ Orbitrap XL or on a ThermoQuest Finnigan LCQ Deca mass spectrometer. Conditions
106 of analysis, ionization method and m/z for all species are given in the Supplementary Material.
107 Data from these analyses also did not indicate molecular ions reported previously by Jaja-
108 Chimedza et al. (2012; 2015) to represent known PMAs, thus suggesting that the investigated
109 strains of *R. raciborskii* and *A. gracile* are not producers of these metabolites. This is the first
110 reported investigation for the presence of PMAs in European cyanobacteria.

111 In conclusion, the findings of the present study, despite being negative, are important in
112 view of the limited information available on the presence PMAs in cyanobacteria, particularly
113 from European freshwaters, and in terms of ecological and health risk assessment if one

considers the disruptive, teratogenic action of these metabolites (Jaja-Chimedza et al. 2012). Our results also indicate that production of PMAs may potentially be geographically diversified with Australian strains of *R. raciborskii* capable of their synthesis (Jaja-Chimedza et al. 2015), on contract to those occurring in Central Europe. One should however note that this is a preliminary study and further investigations screening more diverse strains are required before final conclusions can be drawn in this regard. The exclusion of PMAs production by tested strains does not unambiguously exclude any teratogenic effect, as other cyanobacteria species have been found to produce teratogenic compounds such as retinoic acids and carotenoid glycosides (Wu et al. 2012; Jaja-Chimedza et al. 2017), which were not considered here. Further research is required to elucidate the as yet unknown biosynthesis of PMAs, identify potential factors regulating their synthesis and to evaluate the frequency of occurrence in European cyanobacteria. Moreover, continued study is needed in order to elucidate the toxicity previously observed for selected European strains of *R. raciborskii* as well as teratogenicity of various cyanobacteria species.

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